METHODS AND COMPOSITIONS FOR RESTORING ANTIBIOTIC SUSCEPTIBILITY IN GLYCOPEPTIDE-RESISTANT *ENTEROCOCCUS*

Related Applications

This application claims priority under 35 USC §119(e) from U.S. Provisional Patent Application Serial No. 60/149,313, filed on August 17, 1999, entitled METHODS AND COMPOSITIONS FOR RESTORING ANTIBIOTIC SUSCEPTIBILITY IN GLYCOPEPTIDE-RESISTANT *ENTEROCOCCUS*. The contents of the provisional application are hereby expressly incorporated by reference.

Government Support

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Field of the Invention

This invention relates to methods for reducing antibiotic resistance in vancomycin resistant bacteria.

Background of the Invention

Over the past decade, the emergence of antibiotic-resistant bacteria, particularly multidrug-resistant strains, have created an increasingly concerning clinical dilemma (Gold, et al., *N. Engl. J. Med.*, 1996, 335:1445-1453). Included among these pathogens are enterococci which have developed relative, and in some cases, absolute resistance to the mainstays of antimicrobial therapy, including beta-lactam and aminoglycoside antibiotics, and more recently, the glycopeptide, vancomycin (Eliopoulos, G.M., *Infect. Dis. Clin. North. Am.* 1997;11:851-65). While new pharmacologic agents continue to be developed in order to remedy this therapeutic shortfall, drug resistance and consequential treatment failure to even investigational agents such as the streptogramins in the setting of vancomycin-resistant enterococcal infections highlight the ongoing need for effective, potentially novel means of treating these organisms (Chang, et al., *Diag. Microbiol. Infect. Dis.*, 1999;33:299-303).

Vancomycin Resistant Enterococcus

Enterococci are Gram-positive cocci which, prior to DNA homology studies, were classified as Lancefield group D streptococci (Moellering, R.C. Jr., In:Mandell GL, Bennett JE and Dolin R eds. *Principles and Practices of Infectious Diseases*. New York:Churchhill Livingstone. 1995:1826-1835). While these organisms are known constituents of the gastrointestinal and genital tract bacterial flora, enterococci have rapidly emerged as clinically relevant pathogens especially in the nosocomial setting. In fact, enterococci are the second most common cause of nosocomial infections in the United States as well as a frequent cause of nosocomial bacteremia (Eliopoulos, G.M., *Infect. Dis. Clin. North. Am.* 1997;11:851-65); Schaberg, et al., *Am. J. Med.*, 1991:91(3B):72S-85S). Far from being inconsequential, the mortality attributable to vancomycin resistant enterococcal bacteremia has been estimated to approach 25% in some studies (Edmond, et al., *Clin. Infect. Dis.*, 1996;23:1234-1239).

Vancomycin Mechanism-of-Action

First introduced in the 1950's as a means for treating penicillin-resistant staphylococcal infections, vancomycin, a glycopolypeptide antibiotic, has become the drug-of-choice for the treatment of beta-lactam antibiotic-resistant Gram-positive bacterial infections (Fekety, et al., In: Mandell, et al. *Principles and Practices of Infectious Diseases*. New York:Churchhill Livingstone, 1995;346-354). While other ancillary mechanisms-of-action continue to be investigated, the major mechanism of vancomycin is the inhibition of polymerization and transpeptidation of the bacterial cell wall peptidoglycan (Ge, et al., *Science* 1999;284:507-11). This structure serves an important function in bacteria: the inhibition of osmolysis. In the wildtype enterococci, cell wall production is characterized by peptidoglycan synthesis in which two D-alanines are ligated to form a dipeptide which is then added to the carboxy-terminus of peptidoglycan precursors (Walsh, C.T., *J. Biol. Chem.*, 1989;264:2393-2396). Vancomycin interferes with this process by complexing with the terminal D-alanine residues at the outer portion of the cytoplasmic membrane (Beauregard, et al., *Antimicrob. Agents chemother.*, 1995;39:791-785; Reynolds, et al., *Euro. J. Clin. Microbiol. Intect. Dis.*, 1989;943-950). This blocks subsequent cell wall formation by perturbing the further processing of peptidoglycan precursors by transglycosidases. Vancomycin also blocks catalysis by enterococcal transpeptidases and D,D-carboxypeptidases.

Vancomycin Resistance

Several phenotypes of glycopeptide resistance in enterococci have been described (Eliopoulos, G.M., *Infect. Dis. Clin. North. Am.* 1997;11:851-65). Class A glycopeptide resistance (VanA), which was targeted in this study, is found in both the clinically relevant *Enterococcus faecalis* and *Enterococcus faecium* species, and is characterized by high-level vancomycin resistance with MICs $\geq$ 64 μg/mL as well as resistance to teicoplanin, a related glycopeptide antibiotic (Eliopoulos, G.M., *Infect. Dis. Clin. North. Am.* 1997;11:851-65).

The genotypic characterization of Class A vancomycin resistance has uncovered potential targets for gene-based anti-drug resistance determinant strategy. The genetic basis for VanA phenotypic resistance is a transposon-based operon consisting of 7 genes including *vanR, vanS, vanH, vanA, vanX, vanY, and vanZ* (Arthur, et al., *Antimicrob. agent Chemother.*, 1993;37:1563-1571; Bugg, et al., *Biochem.*, 1991;30:2017-2021) (Figure 1). The products of these genes function in concert to negate the inhibitory effects of vancomycin by, in essence, allowing for an alternate biosynthetic pathway for the production of cell wall precursors which less avidly bind vancomycin. The transcription of *vanH, -A, and –X* are under the control of the *vanH* promoter. This promoter is inducible by the binding of the phosphorylated gene product of *vanR* (Arthur, et al., *J. Bacteriol.*, 1992;174:2582-2591; Holman, et al., *Biochem.*, 1994;33:4625-4631).

Therapeutic Gene Transfer Background

In an attempt to inhibit pathogens which are refractory to conventional pharmacological antimicrobial agents, gene-based therapeutics have been studied, though for the most part, in eukaryotic systems. For example, nucleic acid binding decoys, antisense nucleic acids (antisense RNA and DNA), ribozymes, and trans-dominant mutants are among the many gene therapy motifs which have been used to target the expression of key viral functions in human immunodeficiency virus, type 1; human papilloma virus; hepatitis viruses, and Herpesviridae infections (Chatterjee, et al., *Science*, 1992, 258:1485-1488; Weiss, et al., *Cell. Mol. Life. Sci.*, 1999, 55:334-58; Yamada, et al., *Virol.*, 1996, 70:1596-1601; Inouye, et al., *J. Virol.*, 1997, 71(5):4071-4080; Yamamoto, et al., *Hepatology*, 1999, 30:300-307; Shillitoe, et al., *Cancer Gene Ther.*, 1994, 1:193-204; Flores-Aguilar, et al., *J. Infect. Dis.*, 1997, 175:1308-1316) . Additionally, they have been studied for their ability to inhibit pro-oncogenic cellular functions (Mercola, et al., *Cancer Gene Ther.*, 1995, 2:47-59; Seth, et al., *Cancer Gene Ther.*, 1997, 4:383-390; Rubin, et al., *Curr. Opin. Pediatr.*, 1999, 11:39-46).

A cornerstone of a successful gene-based tactic is that the target nucleic acid sequence encode for pivotal, highly conserved pathogenic functions. In eukaryotic viral and oncologic

systems, antisense nucleic acids, for example, have also been specifically used to inhibit the expression of key viral or cellular functional proteins including the expression of drug resistance determinants (Gao, et al., *Anticancer Res.*, 1998, 18:3073-3076; Inouye, et al., *Antiviral Therapy*, 1999, 4 (Supplement 1):121). In comparison, examples of gene-based strategies in prokaryotic systems are scant (Takada-Guerrier, et al., *Proc. Natl. Acad. Sci* USA, 1997;94:8468-8472; White, et al., *Antimicrob. Agent Chem.*, 1997, 41:2699-2704; Rom, et al., *Am. J. Res. Crit. Care. Med.*, 1997, 156:1993-1998; Nielson, et al., *Nat. Biotech.*, 1998, 16:355-358), and in particular, with enterococci or more specifically, with vancomycin-resistant enterococci, have yet to be reported. Although data have been published on the use of anti-resistance determinant genetic elements in other microorganisms (e.g. *Escherichia coli* and *Staphylococcus aureas*) there are yet no published data on the use of this technology for vancomycin-resistant *Enterococcus* (Takada-Guerrier, et al., *Proc. Natl. Acad. Sci USA*, 1997, 94:8468-8472; White, et al., *Antimicrob. Agent Chem.*, 1997, 41:2699-2704).

Summary of the Invention

In the most basic of terms, a successful strategy against antibiotic resistant enterococci would require either (1) the retention of antimicrobial activity despite the presence of the drug resistance mechanism (i.e. a lack of cross-resistance), or (2) the perturbation of the antibiotic resistance mechanism itself and, as a consequence, reversion of the bacterium to a drug-susceptible phenotype. In our studies, the unique approach taken towards the treatment of vancomycin-resistant enterococci is of the latter type. Herein, we present a gene-based strategy which targets a key vancomycin resistance determinant and results in the restoration of vancomycin susceptibility in previously glycopeptide-resistant enterococci.

Thus, the invention overcomes the above-noted and other problems of the prior art by providing methods and related compositions for reducing antibiotic resistance in vancomycin resistant microorganisms. More particularly, the present invention provides a gene cassette comprised of the *vanH* promoter and a single copy of a *vanA* antisense gene in an enterococcal shuttle vector. Using this invention, we have demonstrated an ability to increase the vancomycin susceptibility in previously resistant *Enterococcus faecalis*.

According to one aspect of the invention, a method for reducing vancomycin resistance in a vancomycin-resistant organism is provided. The method involves introducing into the organism at least one "anti-sense vancomycin resistance molecule" under conditions to inhibit expression of a vancomycin resistance gene. By "inhibit expression" it is meant to

inhibit replication, transcription, and/or translation of a vancomycin gene since inhibition of any of these processes results in the inhibition of expression of a protein encoded by a vancomycin gene. Exemplary vancomycin-resistant organisms include the Gram-positive bacteria *Enterococcus faecium* and *Enterococcus faecalis* and other bacteria to which these organisms have the potential of transferring resistance determinants, given that VanA is a transferable form of resistance and that it could be transferred to other clinically significant pathogens such as *Streptococcus Pneumococcus*, and *Staphylococcus*. (See, e.g., Brisson-Noel A., et al., *J. Bacteriol,* 1988, 170:1739-1745).

Preferably, the vancomycin resistant organism is a Gram-positive bacteria and, more preferably, the organism is an *Enterococcus*.

Vancomycin resistance can take a variety of forms depending upon the nature of the gene cluster which mediates the resistance phenotype. Thus, exemplary vancomycin resistant organisms of the invention may exhibit one or more of the following phenotypes: VanA resistance, VanB resistance, VanC resistance, and VanD resistance. VanA resistance is mediated by a gene cluster which includes seven genes: *vanR* (SEQ ID NO:18), *vanS* (SEQ ID NO:19), *vanH* (SEQ ID NO:20), *vanA* (SEQ ID NO:21), *vanX* (SEQ ID NO:22), *vanY* (SEQ ID NO:23), and *vanZ* (SEQ ID NO:24).

In a preferred embodiment in which the vancomycin resistant organism carries a VanA genotype, the antisense vancomycin resistance molecule is selected from the group consisting of antisense molecules which hybridize under stringent conditions to these target genes or to conserved regions of these target genes (e.g., SEQ ID NOS: 5, 6, 7, 8, 9, and 10). As used herein, such antisense molecules to these target genes are referred to as *vanR* antisense molecules, *vanS* anti-sense molecules, *vanH* anti-sense molecules, *vanA* anti-sense molecules, *vanX* anti-sense molecules, *vanY* anti-sense molecules, and *vanZ* anti-sense molecules, respectively. In a particularly preferred embodiment, the organism is a VanA type, and the anti-sense vancomycin resistance molecule hybridizes under stringent conditions to the *vanA* target gene (SEQ ID NO:21), or to a conserved region of the *vanA* gene (e.g., SEQ ID NOs: 7, and 8). In a further preferred embodiment, the organism is a VanA type, and the anti-sense vancomycin resistance molecule hybridizes under stringent conditions to the *vanX* target gene (SEQ ID NO:22), or to a conserved region of the *vanX* gene (e.g., SEQ ID NO:10).

Additionally or alternatively, the vancomycin resistant organism can be a VanB, VanC, and/or VanD type organism and the anti-sense vancomycin resistance molecule is a

nucleic acid molecule which hybridizes under stringent conditions to these target genes (SEQ ID NO:2 is the vanB gene cluster sequence; SEQ ID NO:3 is the vanC gene sequence; SEQ ID NO:4 is the vanD gene cluster sequence) or to conserved regions of these target genes (e.g., SEQ ID NOS: 11, 12, and 13).

In general, the antisense molecules which hybridize to a conserved region of a target vancomycin resistance gene contain from about 18 to about 1500 nucleotides, more preferably from about 10 to about 30 nucleotides, and most preferably from about 20 to about 30 nucleotides.

In general, the anti-sense vancomycin resistance molecules are introduced to the organism by contacting the vancomycin resistant organism with at least one cassette (typically contained in a vector) comprising one or more "anti-sense vancomycin resistance molecules" under conditions to allow the vector to enter the organism and inhibit expression of one or more vancomycin resistance genes. In general, the vector comprises an expression cassette which permits expression of the anti-sense vancomycin resistance molecules in the organism. The preferred vectors are selected from the group consisting of: an enterococcal shuttle vector (e.g., see the Examples), an enterococcal bacteriophage (Merril CR, et al., *Proc Natl Acad Sci USA*, 1996, 93:3188-92); the nucleic acid portion of a peptide nucleic acid molecule (Good L, et al., *Nat Biotechnol,* 1998; 16:355-8); an enterococcal conjugative transposon or pheromone-responsive plasmid (Murray BE, *Emerg Infect Dis,* 1998, 4:37-47).

In certain embodiments such as those described in detail in the Examples, the cassette contains one or more copies of a *vanA* antisense molecule operatively coupled to a promoter, preferably, the same inducible promoter which drives expression of the *vanH*, *vanA*, and *vanX* resistance determinant, e.g., a *VanR*-responsive promoter such as the *vanH* promoter. As used herein, a *VanR*-responsive refers to a promoter which activates transcription in response to binding of a phosphorylated *VanR* protein.

Preferably, the *VanR*-responsive promoter is a *vanH* promoter (P_{vanH}) or a *vanR* promoter (P_{vanR}), each of which directs transcription of the genes of the vancomycin resistance operon found in several species. These *VanR*-responsive promoters activate transcription in response to binding of an activated *VanR* protein. These promoters include, in addition to the *VanR* binding sites, all other sequences required for efficient transcriptional activation of the gene or genes located downstream of the promoters. In general, these *VanR*-responsive promoters (P_{vanH}, P_{vanR}) include the 60 nucleotides immediately upstream (nucleotides -60 to -1) of the genes encoding a *VanR* protein or a *VanR* protein, which

sequences include a *VanR* binding site, and other sites which contribute to efficient *VanR*-responsive activation of gene transcription.

Other *VanR*-responsive promoters can be used to effect transcription of protein coding sequences. For example, alternative *VanR*-responsive promoters can be identified by searching databases of bacterial nucleotide sequences for sequences which have *VanR* binding sites in proximity to sites which contribute to efficient bacterial transcriptional activation, e.g. a consensus binding site for bacterial DNA polymerase. Such sites are well-known to one of ordinary skill in the art. *VanR*-responsive promoters can also be identified by genetic screening and cloning protocols that are standard in the art, as described in Sambrook. Further, non-natural *VanR* promoters can be prepared by combining a *VanR* binding site with the other nucleotide sequences which contribute to efficient bacterial transcriptional activity. Such synthetic or non-natural *VanR*-responsive promoters can be synthesized directly by chemical means, such as by use of an automated DNA synthesizer.

In an analogous manner, other embodiments can be prepared in which the expression cassette contains one or more copies of a different vancomycin resistance antisense molecule operatively coupled to a promoter which drives expression of the targeted antisense gene.

In yet another aspect of the invention, an alternative method for reducing vancomycin resistance is provided. According to this aspect of the invention, the method involves enhancing expression of a *VanR*-responsive promoter, such as a *vanH* promoter, in the organism to an amount sufficient to reduce vancomycin resistance in the organism, wherein the *vanH* promoter is not operatively coupled to a vancomycin resistance gene of the organism. As used herein, a "vancomycin resistance gene of the organism" refers to the gene in its native configuration contained within the genome of the organism, i.e., not isolated from the organism.

In certain preferred embodiments, the *vanH* promoter is operatively coupled to an antisense vancomycin resistance molecule, such as a *vanA* anti-sense molecule. More preferably, the *vanH* promoter (alone or operatively coupled to an antisense vancomycin resistance molecule) is contained in a cassette. Typically, the cassette is contained in a vector to facilitate transport into and out of the resistant organism. In a particularly preferred embodiment, the vector is an *enterococcal* vector and enhancing expression of the *vanH* promoter involves introducing the vector into the organism. Although not wishing to be bound to a particular theory or mechanism, it is believed that introducing the vector into the

organism results in expression of an amount of the *vanH* promoter sufficient that is sufficient to bind to phosphorylated *VanR* and thereby reduce vancomycin resistance in the organism. In further preferred embodiments, the *VanR*-responsive promoter, such as a *vanH* promoter is co-administered into the organism together with an antisense vancomycin resistance molecule operatively coupled to a *vanH* promoter.

According to still other aspects of the invention, compositions for use in accordance with the methods of the invention are provided. In certain embodiments, the compositions of the invention are isolated nucleic acids that hybridize under stringent conditions to a targeted vancomycin gene or a conserved region thereof, such as described in more detail below. In a particularly preferred embodiment, the isolated nucleic acid is vancomycin resistance gene sequence which has been cloned in the opposite direction (see, e.g., the Examples). Exemplary target genes and conserved regions thereof include the genes which are contained in the VanA resistance gene cluster (GenBank Accession No. M97297, SEQ ID NO:1), the VanB resistance gene cluster (GenBank Accession No. U35369, SEQ ID NO:2), the VanC resistance gene cluster (GenBank Accession No. L29638, SEQ ID NO:3), and the VanD resistance gene cluster (GenBank Accession No. AF130997, SEQ ID NO:4). The location of the individual genes in each gene cluster is set forth in each GenBank listing. Thus, the anti-sense molecules of the invention have sequences which are complementary, and therefore capable of hybridizing to the target genes identified herein, as well as to conserved and/or unique regions of these genes (e.g., by using routine skill to search nucleic acid databases such as GenBank to identify regions of the vancomycin resistance genes which are conserved and/or which are unique). In certain preferred embodiments, the anti-sense molecules of the invention hybridize to regions of the target gene which encode an active site or other which encodes an active site or other functional portion of the encoded protein (e.g., the active site of the ligase encoded by the vanA gene). Using such techniques, Applicants have identified the following nucleotide regions of representative target genes to which the anti-sense molecules can be designed to hybridize (i.e., the anti-sense molecules have complementary nucleotide sequences to the target genes or the selected regions).

SUMMARY TABLE

SEQ ID NO	GENE/ACC NO	NUCLEOTIDE NOS	TARGETED SEQ NO
5	*vanS*/M97297	5657 to 5684	5'-ggtggcgcgggacttggatggcgattg-3'
6	*vanR*/M97297	4258 to 4287	5'ggcgcggatgattatataacgaagcccttt-3'

7	*vanA*/M97297	7719 to 7736	5'-cgagccggaaaaaggctc-3'
8	*vanA*/M97297	7339 to 7358	5'-ggctgcgatattcaaagctc-3'
9	*vanH*/M97297	6033 to 6059	5'-attactgtttatggatgtgagcaggat-3'
10	*vanX*/M97297	8343 to 8368	5'-gtggcttcaaaatcaagccatagccg-3'
11	VanB/U35369	5708 to 5725	5'-cgagccggaaaaaggctc-3'
12	VanB/U35369	5328 to 5347	5'-ggctgcgatattcaaagctc-3'
13	VanD/AF130997	4443 to 4462	5'-ggctgcgatattcaaagctc-3'

It will be understood that anti-sense molecules which contain a few nucleotide residues (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) which hybridize to either side of the above-identified conserved nucleotide regions are embraced within the meaning of the anti-sense molecules disclosed and claimed herein for use in accordance with the methods of the invention.

According to still other aspects of the invention, cassettes containing the isolated nucleic acids of the invention, as well as vectors containing such nucleic acids and/or cassettes, also are provided. Preferably the cassettes further comprise a vancomycin-inducible promoter (e.g., a *VanR*-responsive promoter such as a *vanH* promoter) operatively coupled to one or more isolated nucleic acid molecules of the invention. In still other embodiments, isolated vancomycin resistant organisms containing any of the foregoing isolated nucleic acids, cassettes and/or vectors also are provided.

These and other embodiments and utilities of the invention will become more apparent in reference to the following drawings and detailed description of the preferred embodiments.

All references are incorporated in their entirety herein by reference.

Brief Description of the Sequences

SEQ ID NO:1 -- The nucleic acid encoding the VanA resistance gene cluster of *Enterococcus faecium*. GenBank accession number M97297.

SEQ ID NO:2 -- The nucleic acid encoding the VanB resistance gene cluster of *Enterococcus faecalis*. GenBank accession number U35369.

SEQ ID NO:3 -- The nucleic acid encoding the VanC resistance gene cluster of *Enterococcus casseliflavus*. GenBank accession number L29638.

SEQ ID NO:4 -- The nucleic acid encoding the VanD resistance gene cluster of *Enterococcus faecium*. GenBank accession number AF130997.

SEQ ID NO:5 -- A conserved nucleic acid region of the *vanS* gene of the VanA gene cluster.

SEQ ID NO:6 -- A conserved nucleic acid region of the *vanR* gene of the VanA gene cluster.

SEQ ID NO:7 -- A conserved nucleic acid region of the *vanA* gene of the VanA gene cluster (nucleotides 7719 to 7736).

SEQ ID NO:8 -- A conserved nucleic acid region of the *vanA* gene of the VanA gene cluster (nucleotides 7339 to 7358).

SEQ ID NO:9 -- A conserved nucleic acid region of the *vanH* gene of the VanA gene cluster.

SEQ ID NO:10 -- A conserved nucleic acid region of the *vanX* gene of the VanA gene cluster.

SEQ ID NO:11 -- A conserved nucleic acid region of the vanB gene cluster (nucleotides 5708 to 5725).

SEQ ID NO:12 -- A conserved nucleic acid region of the vanB gene cluster (nucleotides 5328 to 5347).

SEQ ID NO:13 -- A conserved nucleic acid region of the vanD gene cluster.

SEQ ID NO:14 -- A 5' -PCR primer oligonucleotide sequence for the *vanH* promoter, used in conjunction with the primer of SEQ ID NO:15.

SEQ ID NO:15 -- A 3' -PCR primer oligonucleotide sequence for the *vanH* promoter, used in conjunction with the primer of SEQ ID NO:14.

SEQ ID NO:16 -- A 5' -PCR primer oligonucleotide sequence for the *vanA* gene, used in conjunction with the primer of SEQ ID NO:17.

SEQ ID NO:17 -- A 3' -PCR primer oligonucleotide sequence for the *vanA* gene, used in conjunction with the primer of SEQ ID NO:16.

SEQ ID NO:18 -- The nucleotide sequence of the *vanR* gene of the VanA gene cluster (SEQ ID NO:1).

SEQ ID NO:19 -- The nucleotide sequence of the *vanS* gene of the VanA gene cluster (SEQ ID NO:1).

SEQ ID NO:20 -- The nucleotide sequence of the *vanH* gene of the VanA gene cluster (SEQ ID NO:1).

SEQ ID NO:21 -- The nucleotide sequence of the *vanA* gene of the VanA gene cluster (SEQ ID NO:1).

SEQ ID NO:22 -- The nucleotide sequence of the *vanX* gene of the VanA gene cluster (SEQ ID NO:1).

SEQ ID NO:23 -- The nucleotide sequence of the *vanY* gene of the VanA gene cluster (SEQ ID NO:1).

SEQ ID NO:24 -- The nucleotide sequence of the *vanZ* gene of the VanA gene cluster (SEQ ID NO:1).

SEQ ID NO:25 -- A 3' -PCR primer oligonucleotide sequence for the *vanA* gene, used in conjunction with the primer of SEQ ID NO:16.

SEQ ID NO:26 -- The nucleotide sequence of the *vanRB* gene of the VanB gene cluster (SEQ ID NO:2).

SEQ ID NO:27 -- The nucleotide sequence of the *vanSB* gene of the VanB gene cluster (SEQ ID NO:2).

SEQ ID NO:28 -- The nucleotide sequence of the *vanYB* gene of the VanB gene cluster (SEQ ID NO:2).

SEQ ID NO:29 -- The nucleotide sequence of the *vanHB* gene of the VanB gene cluster (SEQ ID NO:2).

SEQ ID NO:30 -- The nucleotide sequence of the *vanB* gene of the VanB gene cluster (SEQ ID NO:2).

SEQ ID NO:31 -- The nucleotide sequence of the *vanXB* gene of the VanB gene cluster (SEQ ID NO:2).

SEQ ID NO:32 -- The nucleotide sequence of the *vanW* gene of the VanB gene cluster (SEQ ID NO:2).

SEQ ID NO:33 -- The nucleotide sequence of the *vanC-2* gene of the VanC gene cluster (SEQ ID NO:3).

SEQ ID NO:34 -- The nucleotide sequence of the *vanRD* gene of the VanD gene cluster (SEQ ID NO:4).

SEQ ID NO:35 -- The nucleotide sequence of the *vanSD* gene of the VanD gene cluster (SEQ ID NO:4).

SEQ ID NO:36 -- The nucleotide sequence of the *vanYD* gene of the VanD gene cluster (SEQ ID NO:4).

SEQ ID NO:37 -- The nucleotide sequence of the *vanHD* gene of the VanD gene cluster (SEQ ID NO:4).

SEQ ID NO:38 -- The nucleotide sequence of the *vanD* gene of the VanD gene cluster (SEQ ID NO:4).

SEQ ID NO:39 -- The nucleotide sequence of the *vanXD* gene of the VanD gene cluster (SEQ ID NO:4).

Brief Description of the Drawings

Figure 1. A schematic showing the organization of genes in the VanA vancomycin resistance operon.

Figure 2. Schematic maps of the shuttle vectors and relevant cloning sites; Fig. 2A shows the parent vector, pAM401; Fig. 2B shows the restriction sites for the *vanH* promoter insertion into pAM401; Fig. 2C shows the restriction sites for the *vanA* antisense insertion *into vanH* promoter/pAM401 construct.

Figure 3. A schematic showing the proposed nucleic acid binding decoy mechanism with the introduction of a shuttle vector carrying the *vanH* promoter alone.

Figure 4. A schematic of the proposed mechanism-of-action of the pAM401-*vanH* promoter-*vanA* antisense recombinant shuttle vector.

Detailed Description of the Invention

While vancomycin has been the mainstay of treatment for beta-lactam antibiotic-resistant enterococci, the increasing prevalence of vancomycin-resistant enterococci has prompted a continued search for new therapeutic approaches. In eukaryotic and prokaryotic systems, gene transfer has been used to define molecular pathogenesis as well as applied towards therapeutic ends. The elucidation of the genetic basis for vancomycin resistance has uncovered potential targets for a unique anti-drug resistance gene-based strategy. Particularly, the preferred embodiments of the present invention consist of a gene cassette comprised of the enterococcal *vanH* promoter and a single copy of a *vanA* antisense gene in the shuttle vector, pAM401. Using this invention, we have demonstrated the ability to increase the vancomycin susceptibility of a vancomycin-resistant *Enterococcus faecalis* by up to 32-fold.

According to one aspect of the invention, a method for reducing vancomycin resistance in a vancomycin-resistant organism is provided. The method involves introducing into the organism at least one "anti-sense vancomycin resistance molecule" under conditions to inhibit expression of a vancomycin resistance gene.

As used herein, "reducing vancomycin resistance" refers to enhancing the susceptibility of a vancomycin resistant organism to vancomycin to a statistically significant extent. In the embodiments illustrated in the Examples, the methods of the invention have

been used to increase the vancomycin susceptibility of a vancomycin-resistant *Enterococcus faecalis* by at least about 16-fold and up to about 32-fold compared to organisms which have not been so treated. These results demonstrate the utility of the invention for reducing vancomycin resistance in the particular organisms tested, as well as the feasibility of using the methods of the invention for treating other types of glycopeptide-resistant bacteria (e.g., VanB, VanC, and VanD type bacteria).

According to certain aspects of the invention, the methods involve inhibiting expression of a vancomycin resistance gene. As used herein, "inhibit expression" refers to inhibiting (i.e., reducing to a detectable extent) replication, transcription, and/or translation of a vancomycin gene since inhibition of any of these processes results in the inhibition of expression of a protein encoded by a vancomycin gene. Exemplary vancomycin-resistant organisms include the Gram-positive bacteria *Enterococcus faecium* and *Enterococcus faecalis* and other bacteria to which these organisms have the potential of transferring resistance determinants, given that VanA is a transferable form of resistance and that it could be transferred to other clinically significant pathogens such as *Streptococcus* species *Pneumococcus*, and *Staphylococcus* species. (See, e.g., Brisson-Noel A. Arthur, M. Courvalin P., "Evidence for natural gene transfer from Gram-positive cocci to *Escherichia coli*," *J. Bacteriol* 170:1739-1745, 1988).

Preferably, the vancomycin resistant organism is a Gram-positive bacteria and, more preferably, the organism is an *Enterococcus*.

Vancomycin resistance can take a variety of forms depending upon the nature of the gene(s) which mediates the resistance phenotype. Thus, exemplary vancomycin resistant organisms of the invention may exhibit one or more of the following phenotypes: VanA resistance, VanB resistance, VanC resistance, and VanD resistance.

VanA resistance is mediated by a gene cluster (SEQ ID NO:1) which includes seven genes: *vanR* (SEQ ID NO:18), *vanS* (SEQ ID NO:19), *vanH* (SEQ ID NO:20), *vanA* (SEQ ID NO:21), *vanX* (SEQ ID NO:22), *vanY* (SEQ ID NO:23), and *vanZ* (SEQ ID NO:24), as described in GenBank Accession No. M97297 (SEQ ID NO:1). VanB resistance is mediated by a gene cluster which includes seven genes: *vanRB* (SEQ ID NO:26), *vanSB* (SEQ ID NO:27), *vanYB* (SEQ ID NO:28), *vanHB* (SEQ ID NO:29), *vanB* (SEQ ID NO:30), *vanXB* (SEQ ID NO:31), and *vanW* (SEQ ID NO:32), as described in GenBank Accession No. U35369 (SEQ ID NO:2); VanC resistance is mediated by a *vanC-2* gene (SEQ ID NO:33), as described in GenBank Accession No. L29638 (SEQ ID NO:3); VanD resistance is mediated

by a gene cluster which includes at least six genes: *vanRD* (SEQ ID NO:34), *vanSD* (SEQ ID NO:35), *vanYD* (SEQ ID NO:36), *vanHD* (SEQ ID NO:37), *vanD* (SEQ ID NO:38), and *vanXD* (SEQ ID NO:39), as described in GenBank Accession No. AF130997 (SEQ ID NO:4). Although the Examples illustrate the application of the invention for treating *vanA* resistance, it is to be understood that the invention can be tailored to treating one or more types of antibiotic resistance to a vancomycin antibiotic by selecting antisense molecules and/or appropriate promoters which can be used to reduce expression of the resistance genes in the targeted organism.

In a preferred embodiment in which the vancomycin resistant organism is a VanA organism, the antisense vancomycin resistance molecule is selected from the group consisting of antisense molecules which hybridize under stringent conditions to these target genes or to conserved, unique, or functionally important regions of these target genes as described above. As used herein, such antisense molecules to these target genes are referred to as *vanA* anti-sense molecules, *vanR* antisense molecules, *vanS* anti-sense molecules, *vanH* anti-sense molecules, *vanX* anti-sense molecules, *vanY* anti-sense molecules, and *vanZ* anti-sense molecules, respectively. In a particularly preferred embodiment, the organism carries a VanA phenotype and the anti-sense vancomycin resistance molecule hybridizes under physiological conditions to the *vanA* target gene or to a conserved region of the *vanA* gene.

Additionally or alternatively, the vancomycin-resistant organism can be a VanB, VanC, and/or VanD resistant organism and the anti-sense vancomycin resistance molecule is selected which hybridizes under stringent conditions to these target genes (SEQ ID NO:2 is the VanB gene cluster sequence; SEQ ID NO:3 is the VanC gene sequence; SEQ ID NO:4 is the VanD gene cluster sequence) or to conserved regions of these target genes. In general, the antisense molecules are isolated molecules which hybridize to a conserved region of a target vancomycin resistance gene contain from about 18 to about 1500 nucleotides, more preferably from about 10 to about 30 nucleotides, and most preferably, from about 20 to about 30 nucleotides.

The nucleic acid molecules described herein preferably are isolated. As used herein with respect to nucleic acids, the term "isolated" means: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which

5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art. An isolated nucleic acid as used herein is not a naturally occurring chromosome.

As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to a messenger RNA (mRNA) transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under the physiological conditions of the target organism, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon the sequences of nucleic acids encoding the vancomycin resistance proteins, or upon allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7 bases in length have been used successfully as antisense oligonucleotides. (Wagner et al., *Nature Biotechnol.* 14:840-844, 1996). Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases.

Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in preferred embodiments the antisense oligonuleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions may be targeted. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol. Neurobiol.* 1994, 14(5):439-457) and at which proteins are not expected to bind. Finally, although the listed sequences may include cDNA sequences, one of ordinary skill in the art may easily derive the genomic DNA corresponding to the cDNA of a vancomycin resistance gene. Thus, the present invention also provides for antisense oligonucleotides which are complementary to the genomic DNA corresponding to nucleic acids encoding vancomycin resistance proteins. Similarly, antisense to allelic or homologous cDNAs and genomic DNAs are enabled without undue experimentation.

Exemplary U.S. patents which describe and claim antisense molecules for reducing gene expression include U.S. Patent Nos. 5,734,039; 5,783,683; 5,859,229; 5,858,987; 5,919,677; and 5,916,807; the entire contents of which patents are incorporated in their entirety herein by reference.

In one set of embodiments, the antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments, however, the antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its oligonucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage rather than a phosphodiester linkage between the 5' end of one oligonucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not

normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding vancomycin resistance polypeptides, together with acceptable carriers to deliver these molecules into the target organism.

The compositions of the invention may be administered as part of a pharmaceutical composition to a mammal (e.g., humans, domestic animals, such as dogs, cats, livestock, such as horses, sheep, cows, pigs) hosting a vancomycin resistant organism. Such a pharmaceutical composition may include the antisense oligonucleotides in combination with any standard physiologically and/or pharmaceutically effective amount of the antisense oligonucleotides in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the rout of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art, as further described below.

The compositions of the invention also may be introduced into vancomycin resistant organisms which is ex vivo, i.e., not contained within a mammal. For example, the applications of such compositions include both treatment of vancomycin-resistant enterococci or other clinically significant pathogen infections and colonization including, for example: (1) *ex vivo* eradication of vancomycin-resistant enterococci from frequently colonized settings

(e.g., intensive care units, hemodialysis units, chronic care facilities); (2) *in vivo* clearance of vancomycin-resistant enterococci from colonized gastrointestinal or genitourinary tracts of human and animal subjects; and (3) primary or adjuvant therapy for vancomycin-resistant enterococcal infections. In certain embodiments, antisense oligonucleotides (e.g., a synthetic antisense DNA strand) are used as a means for delivering this motif into bacteria by delivering the genes which code for antisense RNA (e.g., by conjugation, transformation, or transduction with bacteriophage). Accordingly, the antisense motif and other anti-resistance determinant genetic elements of the invention (e.g., nucleic acid binding decoys, transdominant mutants, suicide genes, ribozymes etc.) may be introduced into enterococci via transconjugation or via recombinant bacteriophage.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host organism. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids, phagemids and virus genomes. A cloning vector is one which is able to replicate autonomously or integrated in the genome or host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence by be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose activities are detectable by standard assays known in the art (e.g., β-galactosidase, luciferase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques (e.g., green fluorescent protein). Preferred vectors are those capable of autonomous replication and

expression of the structural gene products present in the DNA segments to which they are operably joined.

As used herein, a coding sequence and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream activator sequences as desired. The vectors of the invention may optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding a vancomycin polypeptide or fragment or variant thereof. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host bacterium.

The vancomycin resistance operons of a targeted organism include, e.g., the naturally occurring operon of *Enterococcus faecium*, or such operons which are substantially identical thereto, e.g., homologs of the vancomycin resistance operon of *Enterococcus faecium* from other species, functionally equivalent variant of the vancomycin resistance operon containing variants of the genes which constitute the naturally occurring operon. Such variants may be sequence variants, e.g., containing conservative substitutions of amino acids and the like as defined herein, or may be different genes which have the same or a similar function as one of the genes found in the naturally-occurring vancomycin operon. For example, the *ddlB* gene of *E. coli* encodes a protein that exhibits similar properties of the VanA protein as discussed below. Thus, a preferred vancomycin resistance operon of a targeted organism typically includes a *vanH* gene, a *ddlB* gene and a *vanX* gene.

The VanA protein product has two activities: a D-Ala-D-hydroxybutyrate depsipeptide ligase activity (Bugg et al., *Biochemistry* 30:2017-2021, 1991). VanA shares 28% amino acid identity with an *E. coli* enzyme, DdlB, which is a D-Ala-D-Ala dipeptide ligase. Two point mutants of DdlB recently have been reported that exhibit depsipeptide ligase activity (S150A and Y126F; Shi & Walsh, *Biochemistry* 34:2768-2776, 1995; Park et al., *Biochemistry*, 1996, *in press*). Thus, these mutants appear to be functional homologs of VanA. Other functional homologs include, for example, genes encoding a VanA or DdlB protein that are present in other vancomycin operons, including such genes present in other species which encode vancomycin resistance. For example, other vancomycin resistant strains of bacteria (i.e., not *Enterococci* which have a VanA operon) have modified Ddl proteins which serve to make depsipeptide termini directly. Non-VanA vancomycin resistance operons such as the VanB vancomycin resistance operon, contain functionally equivalent VanA homologs. Other functional homologs, either natural or non-natural, are also embraced by the invention.

In general, the anti-sense vancomycin resistance molecules are introduced to the organism by contacting the vancomycin resistant organism with at least one cassette, preferably contained in a vector, which cassette comprises one or more "anti-sense vancomycin resistance molecules" operably coupled to a promoter (e.g., a *VanR* response promoter). The cassette is contacted with the organism under conditions which allow the cassette and/or vector to enter the organism and inhibit expression of one or more vancomycin resistance genes. Typically, the vector comprises an expression cassette which permits expression of the anti-sense vancomycin resistance molecules in the organism. The preferred vectors are selected from the group consisting of: an enterococcal shuttle vector

(e.g., see the Examples), an enterococcal bacteriophage (Merril CR, Biswas B, Carlton R, Jensen NC, Creed GJ, Zullo S, Adhya S, "Long-Circulating Bacteriophage as Antibacterial Agents," *Proc Natl Acad Sci USA*, 1996; 93:3188-92); the nucleic acid portion of a peptide nucleic acid molecule (Good L, Nielsen PE, "Antisense Inhibition of Gene Expression in Bacteria by PNA Targeting To mRNA," *Nat Biotechnol* 1998; 16:355-8); an enterococcal conjugative transposon or pheromone-responsive plasmid (Murray BE, "Diversity Among Multidrug-Resistant Enterococci," *Emerg Infect Dis* 1998; 4:37-47).

In certain embodiments such as those described in detail in the Examples, the cassette contains one or more copies of a *vanA* antisense molecule, e.g., in tandem, operatively coupled to a promoter, preferably, the same inducible promoter which drives expression of the *vanA* resistance determinant, e.g., a *VanR*-responsive promoter such as the *vanH* promoter. As used herein, a *VanR*-responsive refers to a promoter which activates transcription in response to binding of an activated *VanR* protein. These promoters include, in addition to the *VanR* binding site, all other sequences required for efficient transcriptional activation of the gene or genes located downstream of the promoters. In an analogous manner, other embodiments can be prepared in which the expression cassette contains one or more copies of a different vancomycin antisense molecule operatively coupled to a promoter which drives expression of the targeted antisense gene.

In yet another aspect of the invention, an alternative method for reducing vancomycin resistance is provided. According to this aspect of the invention, the method involves enhancing expression of a *VanR*-responsive promoter (e.g., a *vanH* promoter) in the organism to an amount sufficient to reduce vancomycin resistance in the organism, wherein the *vanH* promoter is not operatively coupled to a vancomycin resistance gene of the organism. As used herein, a "vancomycin resistance gene of the organism" refers to the gene in its native configuration contained within the genome of the organism, i.e., not isolated from the organism or attached to nucleic acid which is not contained within the genome of the organism.

In certain preferred embodiments, the *VanR*-responsive promoter is operatively coupled to an antisense vancomycin resistance molecule, such as a *vanA* anti-sense molecule. More preferably, the *VanR*-responsive promoter (alone or operatively coupled to an antisense vancomycin resistance molecule) is contained in a cassette. Typically, the cassette is contained in a vector to facilitate transport into and out of the resistant organism. In a particularly preferred embodiment, the vector is an *enterococcal* vector and enhancing

expression of the *VanR*-responsive promoter involves introducing the vector into the organism. An exemplary cassette, vector and process for introducing the cassette into a vancomycin resistant organism and representative experimental evidence showing the efficacy of the claimed methods for reducing antibiotic resistance in a vancomycin resistant organism are described in the Examples.

Although not wishing to be bound to a particular theory or mechanism, it is believed that introducing the vector into the organism results in expression of an amount of the *VanR*-responsive promoter (e.g., a *vanH* promoter) that is sufficient to bind to phosphorylated *VanR* and thereby reduce vancomycin resistance in the organism by competitively sequestering the phosphorylated *VanR* protein.

According to still other aspects of the invention, compositions for use in accordance with the methods of the invention are provided. In certain embodiments, the compositions of the invention are isolated nucleic acids that hybridize under stringent conditions to a targeted vancomycin gene or a conserved region thereof, such as described in more detail below. In a particularly preferred embodiment, the isolated nucleic acid is vancomycin resistance gene sequence which has been cloned in the opposite direction (see, e.g., the Examples). Exemplary target genes and conserved regions thereof include the genes which are contained in the *vanA* resistance gene cluster (GenBank Accession No. M97297, SEQ ID NO:1), the *vanB* resistance gene cluster (GenBank Accession No. U35369, SEQ ID NO:2), the *vanC* resistance gene (GenBank Accession No. L29638, SEQ ID NO:3), and the *vanD* resistance gene cluster (GenBank Accession No. AF130997, SEQ ID NO:4). The location of the individual genes in each gene cluster is set forth in each GenBank listing. Thus, the anti-sense molecules of the invention have sequences which are complementary, and therefore capable of hybridizing to the target genes identified herein, as well as to conserved and/or unique regions of these genes (e.g., by using routine skill to search nucleic acid databases such as GenBank to identify regions of the vancomycin resistance genes which are conserved and/or which are unique). In certain preferred embodiments, the anti-sense molecules of the invention hybridize to regions of the target gene which encode an active site or other which encodes an active site or other functional portion of the encoded protein (e.g., the active site of the ligase encoded by the *vanA* gene). Using such techniques, Applicants have identified the following nucleotide regions of representative target genes to which the anti-sense molecules can be designed to hybridize (i.e., the anti-sense molecules have complementary nucleotide sequences to the target genes or the selected regions).

SUMMARY TABLE

SEQ ID NO	GENE/ACC NO	NUCLEOTIDE NOS	TARGETED SEQ NO
5	*vanS*/M97297	5657 to 5684	5'-ggtggcgcgggacttggatggcgattg-3'
6	*vanR*/M97297	4258 to 4287	5'ggcgcggatgattatataacgaagccctttt-3'
7	*vanA*/M97297	7719 to 7736	5'-cgagccggaaaaaggctc-3'
8	*vanA*/M97297	7339 to 7358	5'-ggctgcgatattcaaagctc-3'
9	*vanH*/M97297	6033 to 6059	5'-attactgtttatggatgtgagcaggat-3'
10	*vanX*/M97297	8343 to 8368	5'-gtggcttcaaaatcaagccatagccg-3'
11	vanB/U35369	5708 to 5725	5'-cgagccggaaaaaggctc-3'
12	vanB/U35369	5328 to 5347	5'-ggctgcgatattcaaagctc-3'
13	vanD/AF130997	4443 to 4462	5'-ggctgcgatattcaaagctc-3'

It will be understood that anti-sense molecules which contain a few nucleotide residues (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) which hybridize to either side of the above-identified conserved nucleotide regions are embraced within the meaning of the anti-sense molecules disclosed and claimed herein for use in accordance with the methods of the invention.

The term "stringent conditions" as used herein refers to parameters with which the art is familiar. More specifically, stringent conditions, as used herein, refers to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH_2PO_4 (pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 x SSC/0.1 x SDS at 65°C.

There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of homologs and alleles of nucleic acids encoding proteins of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

According to still other aspects of the invention, cassettes containing the isolated nucleic acids of the invention, as well as vectors containing such nucleic acids and/or cassettes, also are provided. Preferably the cassettes further comprise a vancomycin-inducible promoter (e.g., a *VanR*-responsive promoter such as a *vanH* promoter) operatively coupled to one or more isolated nucleic acid molecules of the invention. In still other embodiments, isolated vancomycin resistant organisms containing any of the foregoing isolated nucleic acids, cassettes and/or vectors also are provided.

"Co-administering," as used herein, refers to administering simultaneously two or more compounds (constructs) of the invention (e.g., the *VanR*-responsive promoter, such as a *vanH* promoter, and an antisense vancomycin resistance molecule operatively coupled to a *vanH* promoter), as an admixture in a single composition, or sequentially, close enough in time so that the compounds may exert an additive or even synergistic effect, i.e., on reducing vancomycin resistance.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

Examples

Plasmids

The parent shuttle plasmid used in the test vector constructs was pAM401 (American Type Culture Collection, Rockville, MD) (Wirth, et al., *J. Bacteriol.*, 1986;165:831-836) . This plasmid is a high copy shuttle vector containing both Gram-negative bacillary (*Eschericia coli*) and enterococcal (*Enterococcus faecalis*) elements necessary for replication in these two bacterial types (Figure 2). To aid in selection of appropriately transformed clones, this plasmid also contains tetracycline and chloramphenicol resistance genes.

The cloning vector, pAMP1 (Gibco BRL, Rockville, MD), was also employed for the cloning of polymerase chain reaction-amplified fragments.

Construction of Recombinant Enterococcal Shuttle Vectors

The structures of the recombinant pAM401 shuttle vectors, including their pertinent restriction sites and vector constituents, are outlined in Figure 2 (Wirth, et al., *J Bacteriol,* 1986, 165:831-6). To construct a pAM401 shuttle vector containing the *vanH* promoter alone, *vanHP* was removed from pAMP1-*vanHP* using Xba I and Sal I restriction enzymes and ligated into pAM401 pre-digested with the same enzymes with the resultant pAM401-*vanHP*

shuttle vector (Figure 2). To produce the pAM401-*vanHP-vanA* antisense, *vanA* was digested out of pAMP1-*vanA* antisense with Xho I and Sal I and cloned into the Sal I site in pAM401-*vanHP* in the anti-coding direction.

Bacterial Strains

Vancomycin-resistant *Enterococcus faecalis* strains, designated A407 and A403, were VanA phenotype clinical isolates obtained from E. Cercenada (Hospital General Gregorio Marañón, Madrid, Spain). A1221 is a VanA strain of *Enterococcus faecium* resulting from the transconjugation with a VanA strain of *Enterococcus faecalis* (A312) obtained from F. Tenover (Centers for Disease Control, Atlanta, GA). These strains were identified as *Enterococcus faecalis* or *faecium* by the use of API-Rapid Strep Strips (bioMeriux Vitex, Inc., Hazelwood, MO). The presence of the *vanA* genotype was confirmed by DNA probe analysis as previously described (Eliopoulos, et al., *Antimicrob. Agents Chemother.*, 1998, 42:1088-92).

Vancomycin susceptibilities were determined by the National Committee for Clinical Laboratory Standards agar dilution method (National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A4. Wayne, PA: NCCCLS, 1997). Commercially prepared competent DH5-alpha *Eschericia coli* (Gibco BRL, Rockville, MD) were also used in the cloning and sub-cloning of the vectors via a standard transformation protocol (Sambrook, et al., In:*Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1989; 1.74).

Antibiotics, Culture Media, Cloning Reagents

Vancomycin and other antimicrobial agents were purchased from Sigma (St. Louis, MO). Restriction and modifying enzymes were obtained from Promega (Madison, WI) and New England BioLabs, Inc. (Beverly, MA). *Eschericia coli* were grown in Luria-Bertani medium and enterococci were grown on Mueller-Hinton or Blood-agar medium. Plasmid preparations were performed using Promega Wizard DNA Purification systems (Madison, WI).

vanH Promoter and vanA Antisense Construction

An approximate 450 base-pair fragment containing the *vanH* promoter - previously described to be necessary for expression of *vanH, -A,* and *–X* - was amplified using genomic DNA from a known strain of VanA strain *Enterococcus faecium* (A1221) as a template (Arthur, et al., *J. Bacter.*, 1992, 174:2582-2591). 5' and 3' primers were synthesized by

Gibco BRL (Rockville, MD). The primer sequences for the respective 5' and 3' *vanH* promoter primers as follows:

5'-CUA CUA CUA CUA CGA ATT CAA GAA CAC TGG-3' (SEQ ID NO:14)

5'-CAU CAU CAU CAU CCA ACC CTT TCT GTG AAA GGC ACC-3' (SEQ ID NO:15)

Polymerase chain reaction amplification was conducted through the use of a Perkin-Elmer 9600 thermocycler for 30 cycles of 94°C, 55°C, and 72°C for 30 seconds each. The resulting amplification product, termed *vanHP* (*vanH* promoter) was then subcloned into the plasmid, pAMP1 (Gibco BRL, Rockville, MD), using the Cloneamp™ (Gibco BRL, Rockville, MD) cloning protocol.

The *vanA* gene was amplified using the following primer pair and subcloning the product into pAMP1 to create a plasmid designated pAMP1-*vanA* antisense:

5'-CUA CUA CUA CUA CTC GAG GCT TAT CAC CCC TTT AAC GC-3' (SEQ ID NO:16)

5'-CAU CAU CAU CAU GGA GAC AGG AGC ATG AAT AG-3' (SEQ ID NO:17)

The polymerase chain reaction with these primers consisted of 30 cycles of 94° C, 55°C, and 72°C for 35 seconds each.

Enterococcal Electroporation

Transformation of the *Enterococcus faecalis* strains with pAM401, pAM401-*vanHP*, or pAM401-*vanHP-vanA* antisense was accomplished via electroporation with a Biorad Gene Pulser™ (Friesenegger, et al., *FEMS Microbiol. Letter*, 1991;79:323-328) . In this procedure, 40 ul of electrocompetent enterococci were combined in a sterile 0.1 cm electroporation cuvette with 2 μl of purified plasmid DNA. The electroporation apparatus settings were 1.50 volts and 400 ohms. Under these conditions, resultant time constants are typically in the 9 millisecond range.

Vancomycin Susceptibility Assays: Agar and Broth Dilutions

Vancomycin susceptibilities were determined using the standard National Committee for Clinical Laboratory Standards (NCCLS) agar dilution protocol (National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A4. Wayne, PA: NCCCLS, 1997). In this assay, the test antibiotic, in this case, vancomycin, was incorporated into Mueller Hinton II agar medium (Becton Dickenson) at two-fold dilutions ranging from concentrations of 0 μg/ml up to 512 μg /ml. The agar was then poured into respective sterile plates. Bacterial strains were then inoculated onto the agar plates and incubated at 35°C overnight. The

minimum inhibitory concentration (MIC) was then determined by the lowest concentration of antibiotic that completely inhibited colony growth.

Gene Expression-RT-PCR

A single colony of A407 with the pAM401-*vanHP-vanA* antisense construct was grown in brain-heart infusion (BHI) liquid media with sub-inhibitory concentrations of vancomycin (1 μg/ml) and chloramphenicol (10 μg/ml). Bacterial RNA was prepared using the Qiagen RNeasy® protocol for the isolation of total RNA (Qiagen Inc. Valencia, CA) modified to incorporate a step of treatment with RNAse free DNAse applied directly on the QIAamp® column (both Qiagen Inc. Valencia, CA). Then a modified Titan™ One tube RT-PCR protocol (Roche molecular biochemicals, Indianapolis, IN) was followed. The samples were then reverse transcribed and amplified by one-step RT-PCR. Each reaction mix contained template RNA (5μg), enzyme (either Titan enzyme mix, reverse and forward PCR primers and buffer components recommended for optimal enzyme activity. The forward (5'-CUA CUA CUA CUA CTC GAG GCT TAT CAC CCC TTT AAC GC -3' -SEQ ID NO:16) and the reverse primer (5'-CGA ATA CCG CAA GCG ACA G-3' -SEQ ID NO:25) were designed to amplify a 1.1 kb bacterial RNA sequence. The RT reaction was performed at 45°C for 60 min, followed by PCR in a Perkin Elmer Model 9600 Thermal Cycler with the following thermal profile: Initial denaturation: 95°C for 3 min then 35 cycles of denaturation (93°C, 15 s), annealing (55°C, 30 s), elongation (68°C, 70 s) and a final extension step (72°C, 7 min). Amplification products were analyzed by gel electrophoresis.

Results

Changes in Vancomycin Phenotypic Susceptibility

The vancomycin susceptibility of a *vanA Enterococcus faecalis* strain, A407, was assessed after electroporation with either pAM401; pAM401-*vanHP*; or pAM401-*vanHP-vanA* antisense. While the vancomycin minimum inhibitory concentration (MIC) remained at 128 μg/ml in A407 containing the pAM401 shuttle vector alone, the introduction of pAM401 with the *vanH* promoter decreased the vancomycin MIC to 16 – 32 μg/ml. The vancomycin MIC was further decreased in response to the pAM401 containing both the *vanH* promoter and the *vanA* antisense, typically in the 8 μg/ml range.

VanH promoter effect on vancomycin resistance

The p*VanR* binding domain within the *vanH* promoter has previously been characterized and consists of an approximate 80 bp region that is considered to have the capacity to bind multiple p-*VanR* molecules (Holman, et al., *Biochemistry*, 1994, 33:4625-

31). Therefore, it was reasoned that the introduction of an exogenous *vanH* promoter cloned into a recombinant enterococcal shuttle vector could increase the vancomycin susceptibility of a target VanA enterococcal isolate through the binding and sequestration of p*VanR* from the native *vanH* promoter. As an initial test of this hypothesis, pAM401 enterococcal shuttle vectors with or without the *vanH* promoter were constructed and electroporated into a VanA strain of *E. faecalis* (A407). The successful transfer of the vectors by electroporation was confirmed through the purification of shuttle vector plasmids from the transformants followed by restriction digest analysis as well as by dideoxy-sequencing. To confirm that MIC changes in the transformants were not related to the loss of the VanA operon, the retention of the resistance determinant gene cluster was confirmed by the polymerase chain reaction (PCR) amplification of relevant genes.

Using both agar and broth dilution methods to determine antibiotic susceptibilities after shuttle vector electroporation, the vancomycin MIC of A407 enterococci transformed with the shuttle vector containing the *vanH* promoter (pAM401-*vanHP*) demonstrated a four-fold reduction in the MIC from 256 μg/mL to 64 μg/mL. In contrast and as expected, control A407 enterococci transformed with the pAM401 vector alone maintained the baseline (MIC of 256 μg/mL) resistance phenotype.

To further support that the vancomycin-resistance phenotypic changes seen with the transformation of pAM401-*vanHP* were due to a transcriptional activator binding decoy effect, the p*VanR* binding domain portion of the *vanH* promoter was amplified and cloned into pAM401 (pAM401-p*VanR*-BD+). As a control, a shuttle vector containing a mutant p*VanR* binding domain-deficient *vanH* promoter (pAM401-p*VanR*-BD-) was also constructed. Consistent with the phenotypic effects seen with the entire *vanH* promoter, the transfer of the p*VanR* binding domain (pAM401-p*VanR*-BD+) into A407 enterococci similarly resulted in a four-fold decrease in the vancomycin MIC to 64 μg/mL. As predicted, no vancomycin susceptibility change resulted from the introduction of the pAM401-p*VanR*-BD- vector.

Effects of vanH promoter-driven vanA antisense RNA expression

Recombinant pAM401 shuttle vectors were then created which contained a gene cassette consisting of the *vanH* promoter and downstream *vanA* antisense gene (pAM401-*vanHP-vanA* antisense), a configuration in which antisense expression would thus be upregulated in parallel that of the native VanA operon in the presence of vancomycin. A control vector that expressed *vanH* promoter-driven *vanA* sense transcripts was also cloned

(pAM401-*vanHP*-vanA sense) and was electroporated into respective A407 VanA *E. faecalis*. The expression of the *vanH* promoter-*vanA* coding and antisense messenger RNA were confirmed by reverse transcriptase PCR (RT-PCR). In A407 *E. faecalis* electroporated with pAM401-*vanHP-vanA* antisense, the vancomycin MIC was reduced to a susceptible range, from 256 μg/mL to 2 μg/mL. As predicted, the MIC of A407 transformed with pAM401-*vanHP-vanA* sense remained at the baseline level of 256 μg/mL.

Discussion

A gene cassette targeting a key antibiotic resistance determinant of the clinically relevant Gram-positive bacterium, *Enterococcus*, has been constructed and consists of the enterococcal *vanH*-promoter driving the expression of a *vanA* antisense gene introduced in an enterococcal shuttle vector. The target gene, *vanA*, is a highly conserved component of a gene cluster that confers high-level resistance to vancomycin, a pivotal antibiotic used to treat infections caused by *Enterococcus* resistant to beta-lactam antibiotics. The *vanH* promoter employed in this construct is the same inducible enterococcal promoter which drives expression of the *vanA* resistance determinant expression (Figure 3). In such an arrangement, where both the resistance and anti-resistance determinant expression are driven by the same inducible promoter, the enterococcal transcriptional factor, phosphorylated *VanR* (p*VanR*), which induces the *vanH* promoter (Arthur, et al., *J. Bacter.*, 1992, 174:2582-2591), is at the same time, sequestrated from the native *vanH* promoter, but also allows for induction of the anti-*vanA* antisense in parallel with the expression of the *vanHAX*. In short, this gene cassette inhibits vancomycin resistance both by an inducible antisense mechanism as well as by functioning as a transcriptional factor binding decoy (Figure 4). Reflective of such a dual mechanism, recombinant shuttle vectors containing the *vanH* promoter or the p*VanR* binding domain effected a partial restoration of vancomycin susceptibility, while full restoration of vancomycin susceptibility resulted with the introduction of a vector containing both *vanH* promoter and *vanA* antisense gene. More specifically, the introduction of a shuttle vector containing the *vanH* promoter alone into a vancomycin-resistant, *vanA*-containing *Enterococcus faecalis* resulted in up to a 16-fold reduction of the minimum inhibitory concentration for vancomycin while a shuttle vector containing both *vanH* promoter and *vanA* antisense increased vancomycin susceptibility even further (approximately 32-fold).

Given the increasingly important role of drug-resistant Gram-positive bacteria such as vancomycin-resistant *Enterococcus* as a cause of significant human disease, combined with a

dearth of effective pharmacological therapeutic options for this pathogen, novel strategies as described above, have several potential applications for (1) the treatment primary infections (2) the eradication of vancomycin-resistant *Enterococcus* from areas which are frequently colonized (e.g. intensive care units, dialysis units, individual patient's bowel flora, the agricultural setting) and (3) as a laboratory tool for the study of antibiotic resistance gene function and pathogenesis.

Recombinant shuttle vectors which target other genes in the *vanA* operon such as *vanX*, as well as polycistronic vectors which contain genetic elements designed to interfere with multiple VanA operon functions (e.g. *vanA, vanH*, and *vanX*), can be constructed using routine experimentation and no more than ordinary skill in the art. Given that an operon analogous to that associated with the VanA phenotype also forms the genetic basis for class B (VanB) vancomycin resistance, analogous compositions against Class B (VanB), as well as other classes of vancomycin resistance operons and genes can be developed as described above. For example, a *vanX* antisense strategy analogous to the vanA antisense strategy was also tested, resulting in lowering vancomycin MICs to the 2 μg/ml range.

Such compositions optimally include gene delivery systems such as bacteriophage, highly efficient transconjugative plasmids, and peptide-nucleic acids.

Deatailed Description of the Drawings

Figure 1. The VanA vancomycin resistance operon. *vanR* represents a response regulator which, after phosphorylation, activates the *vanH* promoter which results in activation of *vanH, vanA*, and *vanX* transcription; v*anS*, a signal sensor, is responsible for the inducibility of the operon by glycopeptide antibiotics;; the *vanH* gene product is a dehydrogenase that generates lactate from pyruvate; *vanA* codes for a ligase which preferentially synthesizes D-ala-D-lac; *vanX* codes for a dipeptidase which degrades the native D-ala-D-ala produced by the wildtype ligase; *vanY* is a carboxypeptidase which removes terminal alanines; *vanZ* is responsible for increased resistance to teicoplanin.

Figure 2. Maps of the shuttle vectors and relevant cloning sites. (A) The parent vector, pAM401. This vector is composed of both *Enterococcus faecalis* (shaded half on right) and *Eschericia coli* (bold portion on left) components. The *cat* region is the chloramphenicol acetyl-transferase gene. The *tet* region is the tetracycline resistance gene. (B) The *vanH* promoter insertion. (C) The *vanA* antisense insertion.

Figure 3. The proposed nucleic acid binding decoy mechanism by which the observed vancomycin minimum inhibitory concentrations are reduced with the introduction of the pAM401 shuttle vector with the *vanH* promoter alone.

Figure 4. A schematic of the proposed mechanism-of-action of the pAM401-*vanH* promoter-*vanA* antisense recombinant shuttle vector.

All terms used herein have their conventional meaning unless otherwise indicated.

All patents and other documents disclosed in this application are incorporated in their entirety herein by reference.

While the invention has been described with respect to certain embodiments, it should be appreciated that many modifications and changes may be made by those of ordinary skill in the art without departing from the spirit of the invention. It is intended that such modification, changes and equivalents fall within the scope of the following claims.

What is claimed is followed by the Abstract and a Sequence Listing.

We claim: